A STUDY OF THE COMBINED ACTION OF X-RAYS AND OF VITAL STAINS UPON PARAMŒCIA.

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It has been definitely recognized that X-rays exercise injurious effects upon both vegetable and animal forms of life and, moreover, that the adult tissues possess a variable absorption capacity for this form of energy. On the other hand, experiments conducted for the purpose of ascertaining the nature of the reactions of bacteria to X-rays have generally proved negative. In marked contrast to seeming bacterial immunity, unicellular organisms show a marked degree of susceptibility.

During the spring and summer of 1916, through the courtesy of the Research Laboratory of the General Electric Company at Schenectady, the author was enabled to make use of X-ray energy in a prosecution of the study of the action of vital stains upon various forms of animal life. These experiments were begun with paramœcia. The investigation comprised, first, the study of the action of vital stains upon these forms of life; secondly, of the effect of X-rays acting alone; and lastly, a study of the reaction to X-ray energy while under the influence of these vital stains. The stains used for this purpose were Nilblau sulfat (Gruebler), Alizarinblau (S), Trypanblau, Isaminblau, Nilblau chlorhydrat, Trypanrot, Dahlia (Gruebler), Neutral red, Anilin red (Sudan III. oil), Janus green (Eimer and Amend), Methylene blue (B.X.) (Merck). These stains were in the form of dry powders. Solutions were prepared with tap water. It had been previously ascertained that this tap water contained no substances injurious to the organisms either at the time of raying or thereafter. The solutions of the stains were freshly prepared for each experiment. In the majority of instances a stock solution consisting of 0.001 gm. of stain to 200 c.c. of water was used, a subsequent dilution of this stain being made before each experiment. This stock solution was not kept for a period

longer than twenty-four hours. Cultures of the paramœcia were added to graded dilutions of the stain with the object of ascertaining the maximum degree of concentration compatible with the life of the organism. The absorption of the stain by the organism was demonstrable through the appearance of stained globules within the body. It was ascertained early in the experiment that this absorption would progress, if undisturbed, to such a degree that the death of the organism resulted. When this phenomenon was about to ensue the motility of the organism became very sluggish, finally ceasing altogether. The elongated, elliptical body-shape was replaced by a spherical form, while the nucleus quickly became deeply stained. It was found not to be possible to bring about a staining reaction of the nucleus without causing the death of the organism. Indeed, the latter became a safe index of over-absorption of the stain.

For the purpose of testing out the absorption capacity for the stain compatible with the life of the organism, 0.5 c.c. cultures of the paramœcia were placed in ordinary watch glasses, to which were added varying amounts of the solutions of the stains. In the preliminary experiments these proportions ranged from two volumes of culture to twelve volumes of stain, to eight volumes of culture to two of stain. The cultures were then carefully attended during the succeeding days with the view of providing sufficient oxygen and to prevent evaporation. A reference to a typical experiment of May 31, 1916, A.M., will be sufficient to give an exact idea of this procedure. For this experiment a stock solution of neutral red in the strength of 0.001 gm. of the dry stain of 200 c.c. water was used.

With such stains as methylene blue and dahlia the absorption capacity of the organisms was so great that it was difficult even in greatly diluted solutions to prevent the staining of the nucleus. With trypanblau, neutral red, and trypan red, on the other hand, the capacity of the organisms for the stain seemed to have a more definite though not absolutely fixed limit. In these instances it was ascertained that, when the cell-body had taken up its maximum carrying amount of the stain, not injurious to the activity of the organism, subsequent immersion over a relatively long period of time in a very greatly diluted solution

Experiment May 31, 1916, A.M.	Observations of June 1, 1916, A.M.	Observations of June 2, 1916, A.M.
Series No. 1, Culture 2 parts (by volume), Stain 8 parts (by volume)	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.
Series No. 2, Culture 2 parts (by volume), Stain 7 parts (by volume)	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.
Series No. 3, Culture 2 parts (by volume), Stain 6 parts (by volume)	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.
Series No. 4, Culture 2 parts (by volume), Stain 5 parts (by volume)	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.
Series No. 5, Culture 2 parts (by volume), Stain 4 parts (by volume)	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.
Series No. 6, Culture 2 parts (by volume), Stain 2 parts (by volume)	Series No. 6, paramœcia sluggishly active, few motionless with stained nuclei.	Series No. 1, paramœcia spherical in shape, motionless, nucleus stained.
Series No. 7, Culture 3 parts (by volume), Stain 2 parts (by volume)	Series No. 7, all active.	Series No. 7, all active.
Series No. 8, Culture 5 parts (by volume), Stain 2 parts (by volume)	Series No. 7, all active.	Series No. 7, all active.

of the stain did not result apparently in a further absorption. The experiments demonstrated, moreover, that the various stains possessed a differing degree of toxicity for these organisms similar to that which had been noted by Lewis in his study of the action of vital stains on bacteria.

Practically it was found to be more convenient to stain organisms for a period of from one and a half to two hours in a solution of a degree of concentration somewhat greater than that which could be tolerated without injury for a period of twelve hours. At the expiration of this staining period the organisms were transferred to the diluent tap water in the proportion of ten volumes of the water to one volume of the organism and stain and studied during the succeeding days. Through an empirical process of experimentation the approximate degree of concentra-

tion tolerated by the organism followed by a dilution as above was ascertained. In the instance of trypanblau, trypan red, isamine blau, and neutral red, this was found to be eight volumes of the water solution of the stain to two of the culture. The stock solution contained 0.001 gm. of the dry stain to 200 c.c. water. Staining of the paramœcia cultures from one and one half to two hours at the room temperature brought about the appearance within one half hour of a deep globular coloration of the cytoplasm of the organism. The subsequent dilution of this stained culture, through the addition of ten volumes of water to one of the culture, prevented subsequent injury, so that the vitality of the organisms remained unimpaired for a period of eight days. The nucleus did not become stained nor was the motility lessened. Ordinarily, the specimens were not studied for a period longer than eight days. With a few, however, observation at the expiration of fourteen days demonstrated still actively motile paramœcia with unstained nuclei. Beyond these limits no effort was made to ascertain either the varying degrees of rapidity of absorption for the different stains or the exact limit of absorptive capacity of the organisms for any particular stain. The dilutions above-mentioned were arbitrarily selected, since with these as a constant, the most marked reactions to X-ray energy were obtained.

The next step in the experimentation consisted of a determination of the amount of X-ray energy necessary to cause the death of unstained organisms. The source of energy was a special Coolidge tube actuated by 35 K.V. with a current strength of 2.0 milliampere and cooled by an airblast. Since the total diameter of the tube was only 7.0 cm. the paramœcia cultures could be placed in ordinary watch glasses at a distance of 8.5 cm. from the center of the target. They were protected from the heat rays arising from the tube by means of three spaced-layers of carbon paper. Numerous experiments showed that there was hardly any loss through evaporation during the raying.

A quotation from the notes will illustrate the general experimental results observed. On the morning of May 5, 1916, a series (*A*) of six cultures, each consisting of numerous unstained paramœcia, was rayed under the conditions mentioned above

during a continuous period of thirty minutes. At the expiration of this exposure the motility of the organisms had become appreciably slower. No changes, however, could be observed either in the character of the cytoplasm or of the nucleus under a magnification of 400 diameters. On May 7, 1916, the observation was made at 2:30 P.M. that a few of these paramœcia were still active though they had lost a great deal of the rapidity of action of their cilia. The majority were motionless and rested upon the bottom of the glass. These had assumed a spherical shape, while in them there could be detected unmistakable evidences of degeneration of both nuclear and cytoplasmic material. In the unstained specimens these facts were demonstrated through a loss of their translucent character and by the granular appearance of the protoplasm. Series *B* consisted of six cultures of paramœcia which were rayed under conditions similar to those of series *A* for a period of twenty-six consecutive minutes. At the termination of the exposure all of the paramœcia were vigorously active. There was no indication of the slowing down of ciliary motion. A study of the specimens forty-eight hours thereafter demonstrated a still vigorous motility. No dead paramœcia could be found.

By this method of varying the length of the exposure to the X-ray energy, the other conditions of the experiment remaining constant, it was ascertained that continuous raying during a period varying from thirty to forty minutes was necessary to bring about, upon the day following the raying, a complete sessation of motility and unmistakable evidences of degeneration. It was not possible, however, to cause protoplasmic changes immediately following the raying in either the nucleus or the cytoplasm. These could be made out first only after a twenty-four hour interval after the raying had elapsed. Beyond this no further inquiry was made to ascertain the cytological cause of cessation of motility. Similarly through repeated experimentation, it was found that exposures of from ten to twenty minutes duration exercised no effect whatever, either immediately or subsequently, upon either the motility or the vitality of the organisms. A study of these rayed organisms was not conducted, however, throughout a period greater than eight days after the raying.

The next and final step in the experiment consisted of a study of the action of the rays upon stained cultures of paramœcia. The staining of the organisms was carried out according to the method which I have detailed above. The energy utilized was that which has also been given above. A reference to the experiment notes will give an idea of the results obtained.

On May 10, 1916, series *C*, consisting of six paramœcium cultures, was rayed beginning at 2:00 P.M., each for a period of 12.5 consecutive minutes. These cultures had previously been stained for 105 minutes in a trypanblau solution of the strength 0.001 gm. of the dry stain to 220 c.c. tap water, of which mixture four volumes were added to one volume of the culture. The staining was begun at 11:15 A.M., and the raying at 2:00 P.M. At the expiration of the raying period, all of the organisms were very sluggish in action. At the expiration of forty-eight hours all were dead. Controls to these experiments were conducted in which the unstained paramœcia were rayed for an equal time, and paramœcia were stained during an equal time. In both instances the organisms were unaffected. In one series (*B*) the unstained paramœcia were rayed for a period of 12.5 consecutive minutes and in *C* during a period of thirty consecutive minutes. Forty-eight hours afterwards the unstained cultures were still alive and vigorous but in series *C* most of the paramœcia were dead, a few, however, remaining sluggishly active.

By a continuation of the experiment along this general line, through increasing the concentration of the stain, it was found possible to cause the death of the organisms with an exposure varying from 2.5 to 5 minutes. A reference to the experiment notes of May 18, 1916, verifies this fact. On this day series *A*, consisting of six cultures of paramœcia, was stained with trypanblau for two hours (stock solution 0.001 gms. to 200 c.c. tap water, diluted in the proportion of 2 volumes of culture to 8 of stain). These were then rayed for five consecutive minutes, beginning at 10:42 A.M. At 11:00 A.M. a few of the paramœcia were observed to be active though this activity was considerably slowed. The majority, however, were motionless. Forty-eight hours later a still greater number of paramœcia were dead. The nucleus had assumed a dark bluish color, the cytoplasm had

become opaque and deeply blue in color, and the organism had assumed a spherical outline. A few, however, were still sluggishly active. On the succeeding day, these also died. The control specimens which were treated to the stain in the same manner, but which were unrayed, were still alive on May 26, eight days afterward. On May 22, six other cultures, series *F*, were rayed 2.5 consecutive minutes after treatment with trypanblau stain prepared as in series *A*. They had been subjected to this stain for two hours previous to the raying. Very few of these paramœcia were alive on the succeeding day. The majority demonstrated a stained nucleus, spherical outline, and loss of ciliary activity. Controls to this series, however, some of which were rayed unstained for a period of ten minutes, and others stained from 11:15 A.M. to 1:41 P.M., but unrayed, were still alive seven days later, and, judging by their activity, had been unaffected.

In general this is the result of the action of X-rays upon vitally-stained paramœcia. The quantity of energy necessary to inhibit the activity of unstained paramœcia varies from sixty to eighty milliampere minutes under the conditions of technic given above. When the cultures are stained, as with trypanblau in the concentration mentioned, the amount of energy required to inhibit their activity is reduced to from five to ten milliampere minutes. There is however, considerable variation within certain limits in the susceptibility of the cultures to X-ray energy and also in the rapidity with which they absorb stains.

So far as could be observed upon these specimens the cause of death was due to an assumption of stain by the nucleus. This causative agent appears to be constant both in the stained but not rayed specimens and also in the stained and rayed specimens. It would appear as if the nuclear membrane were normally impervious to the colloid stain until the degree of concentration of the stain in the cytoplasm became great enough to overcome this resistance. One might assume that X-rays alter this permeability thus permitting the stains to diffuse readily into the nucleus.

Solutions of trypanblau, trypan red, neutral red, and of dahlia when exposed to X-ray energy are not fluorescent nor is there

a loss of color reaction of solutions from prolonged exposure to the rays. Very careful histological examination under high-power magnification has failed up to the present to detect a change in the color of the absorbed stain after raying, or to give evidence of a redistribution of the stain in the cytoplasm. Moreover, a series of experiments in which normal paramœcia were stained by trypanblau solution which had previously been rayed for periods varying from five to thirty minutes, failed to demonstrate either an injurious effect of the rayed stain upon the organisms or an increased rapidity of absorption by the organisms of that stain. The cause for the increased susceptibility of the organisms to X-ray energy is to be found apparently in some factor which is operative only when X-rays act on the cells in the presence of the stain.